Extended lung expression and increased tissue localization of viral IL-10 with adenoviral gene therapy

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Contributed by Harold S. Ginsberg, October 12, 2000

IL-10 is a pleiotropic cytokine that acts as an important regulator of macrophage, T cell, and natural killer cell functions. Human IL-10 (hIL-10) has both stimulatory and inhibitory effects on a wide variety of cell types. Viral IL-10 (vIL-10) possesses only a subset of hIL-10's activities, predominantly its suppression of cytokine synthesis by T helper type 1 clones. In the present report, we evaluated tissue accumulation and biological activity of hIL-10 and vIL-10 *in vivo* in individual organs by using a first-generation adenoviral (Ad) vector administered intratracheally and intravenously. We report the observation that Ad vectors delivering vIL-10, but not hIL-10, are associated with prolonged expression in the lung (>42 days) when delivered intratracheally. In contrast, there was no prolongation in vIL-10 expression when Ad vectors were intravenously administered, although vIL-10 levels in the tissue, but not serum, were markedly increased relative to hIL-10. Moreover, we report an augmented capacity of expressed vIL-10 versus hIL-10 to suppress the acute inflammatory responses in the lung to intratracheal administration of Ad. These findings confirm fundamental differences in Ad-induced expression of vIL-10 and hIL-10 when administered to the lungs. The results further suggest that Ad vectors expressing vIL-10 may have a role as anti-inflammatory agents in the treatment of acute and chronic lung inflammation.

Interleukin 10 (IL-10) is a pleiotropic cytokine that acts as an important regulator of macrophage, T cell, and natural killer cell functions. Cellular IL-10 (cIL-10), as mouse and human IL-10 (hIL-10) are collectively called, has both stimulatory and inhibitory effects on a wide variety of cell types. cIL-10 inhibits production of the cytokines tumor necrosis factor α (TNF-α), interleukin 1α (IL-1α), and interferon γ (IFNγ) by T helper type 1 clones, which is referred to as its cytokine synthesis inhibitory factor (CSIF) activity (1). Many of the inhibitory effects of cIL-10 can be attributed to inhibition of macrophage and dendritic cell function, and cIL-10 has been shown to prevent antigen-specific T cell proliferation indirectly through inhibition of antigen-presenting capacity of monocytes by down-regulation of class II MHC antigens. (2, 3). Conversely, cIL-10 has been found to stimulate proliferation of thymocytes, mast cells, and B cells (1, 2, 4, 5).

BCRFI, an ORF in the Epstein–Barr virus that is commonly referred to as vIL-10, is thought to be a captured cellular cytokine. It bears an 84% amino acid sequence identity to mature hIL-10, but only a 71% nucleic acid sequence identity (6, 7). Despite this high sequence identity, vIL-10 possesses only a subset of hIL-10's activities, predominantly its CSIF activity, macrophage deactivation, and enhancement of the viability of B cells.

Liu *et al.* (8) have shown through competitive displacement experiments that the affinity of hIL-10 for both the hIL-10 receptor and the mouse IL-10 receptor is at least 1,000-fold greater than vIL-10 for these same receptors. Additionally, neutralizing anti-hIL-10 receptor mAb blocks responses of hIL-10 receptor-transfected and normal cells to both hIL-10 and vIL-10. These results indicate that despite vIL-10's decreased binding affinity for the receptor, the IL-10 receptor is necessary for vIL-10 signaling (8).

Despite extensive *in vitro* studies, there has been little *in vivo* data comparing the expression and biological activity of cIL-10 and vIL-10 when delivered with an adenoviral (Ad) vector. In the present report, we have evaluated tissue accumulation and activity of hIL-10 and vIL-10 *in vivo* in individual organs by using an Ad delivery system administered intratracheally (i.t.) and intravenously (i.v.). In a previous report, we noted that clearance of transgene expression from the lung after Ad instillation was rapid, and it depended on the magnitude of the innate immune response (9). Here, we report the observation that Ad vectors delivering vIL-10, but not hIL-10, are associated with prolonged expression in the lung (in excess of 42 days) when delivered i.t. Moreover, we report an augmented capacity of vIL-10 to suppress inflammatory responses to Ad in the lung after i.t. administration. These findings suggest fundamental differences between vIL-10 and hIL-10 when expressed in the lungs by Ad. The results suggest that Ad vectors expressing vIL-10 may have a role as anti-inflammatory agents in the treatment of acute and chronic lung inflammation.

Materials and Methods

Construction of a Recombinant Ad Expressing β-Galactosidase (β-gal), hIL-10, and vIL-10. A derivative of human Ad serotype 5 (10) was used as the source of viral DNA backbone. The construct was deleted in early (E) region 1, polypeptide IX, and early region 3. Specifically, the vector contains a deletion of base pairs 355 to 3325 to eliminate E1a and E1b functions, a deletion of base pairs 3325 to 4021 to eliminate protein IX function, and a deletion of base pairs 28592 to 30470 to eliminate E3 functions (11).

Recombinant adenoviruses were constructed by using standard homologous recombination methods as described by Graham and Prevec (12). To generate recombinant Ad vectors expressing hIL-10 or vIL-10, cDNA sequences encoding hIL-10 or vIL-10 were isolated from the pDSRG-IL10 or pcDSR-BCRF1 plasmids, respectively (obtained from Kevin

Abbreviations: Ad, adenovirus or adenoviral; β-gal, β-galactosidase; TNF-α, tumor necrosis factor α; IL-1α, interleukin 1α; hIL-10, human IL-10; cIL-10, cellular IL-10; vIL-10, viral IL-10; i.t., intratracheal(ly); i.v., intravenous(ly).

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Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.250489297. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.250489297

Moore, DNAX Research Institute, Palo Alto, CA) (7). A recombinant Ad expressing β-gal gene or buffer alone was used as a control (11).

Animal Preparation. Specific pathogen-free female C57BL/6 mice (Charles River Breeding Laboratories) (20–25 g) were housed in a BSL-2 barrier facility with unlimited chow and water for the duration of the experiments.

Mice were anesthetized with 35 mg/kg body weight of intraperitoneal sodium pentobarbital. A midline incision was made in the neck, the trachea was visualized and cannulated with a sterile 30-gauge needle, and 32 μl of buffer or buffer containing Ad vector (10^{10} particles per animal) was delivered. Mice received an i.t. instillation of 10^{10} particles of an Ad construct expressing β-gal (Ad/β-gal), hIL-10 (Ad/hIL-10), or vIL-10 (Ad/vIL-10).

Mice were killed by cervical dislocation at 1, 3, 5, 7, 9, 14, 21, 28, 35, and 42 days after viral instillation. Blood was collected by means of a retro-orbital venipuncture using a capillary tube. Lungs and trachea were removed *en bloc* and snap frozen in liquid nitrogen.

Intravenous tail vein injections of 100 μl of buffer or buffer containing 10^{10} particles of either Ad/hIL-10 or Ad/vIL-10 were also performed, using a sterile 27-gauge needle. Mice were killed as above, blood was collected, and lungs and liver were removed and snap frozen in liquid nitrogen. Alternatively, mice underwent i.v. injection of 20 ng/g of body weight of recombinant hIL-10 (Schering–Plough Research Laboratories, Kenilworth, NJ) or recombinant vIL-10 (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA), and blood was collected at 2, 10, 30, and 60 min after injection.

***In Vitro* Studies.** Murine B10.D2-derived KD2SV cells (obtained from Barbara Knowles, The Jackson Laboratory, Bar Harbor, ME) (13) were transduced overnight with various concentrations of Ad/hIL-10 or Ad/vIL-10. The supernatant was collected at 48 h and the cells were treated with trypsin and harvested. The cell pellet was collected from the washed cells and frozen at −80°C. Cell lysates were generated by homogenization of the cell pellet in a potassium phosphate buffer followed by sonication, centrifugation, and collection of the cell lysate fraction. The supernatants and clarified lysates were assayed for hIL-10 and vIL-10 as described below.

Analytical Methods. β-gal activity was detected in the lung and liver by using a chemiluminescent reporter gene assay system (Tropix, Bedford, MA), as previously reported (9). Bioactive TNF-α was measured in serum and lung and liver homogenates by using the TNF-α-sensitive WEHI 164 clone 13 murine fibrosarcoma cell line (14). Murine IL-6, IL-1α, IL-10, hIL-10, and vIL-10 levels in the organ homogenates, and in the serum, were measured by sandwich ELISA using commercially available reagents (murine IL-6, hIL-10, and vIL-10 by Endogen, Woburn, MA, and murine IL-1α and IL-10 by R&D Systems, Minneapolis, MN).

Pulmonary and hepatic neutrophil sequestration was quantitated by measuring tissue myeloperoxidase content (9). To determine the magnitude of the humoral response that develops against the expressed β-gal, hIL-10, vIL-10, and Ad proteins, a direct ELISA was performed (9, 15). A functional assay to determine the neutralizing capacity of antisera to prevent the capacity of recombinant Ad vectors to infect and transduce HeLa cells was performed as previously reported (9).

Statistical Analysis. Data are presented as the mean ± SEM and $n = 8$–12 for each group. Student's t test was used for analyses comparing two different groups. A one-way analysis of variance (ANOVA) was used to compare animals at different time points receiving the same treatment, and *post hoc* comparisons were performed with Dunn's multiple-range test. A two-way ANOVA was used to evaluate differences between treatment and time, and a *post hoc* comparison was undertaken with a Student–Newman–Keuls multiple-range test. Statistical significance was considered to be achieved if $P < 0.05$.

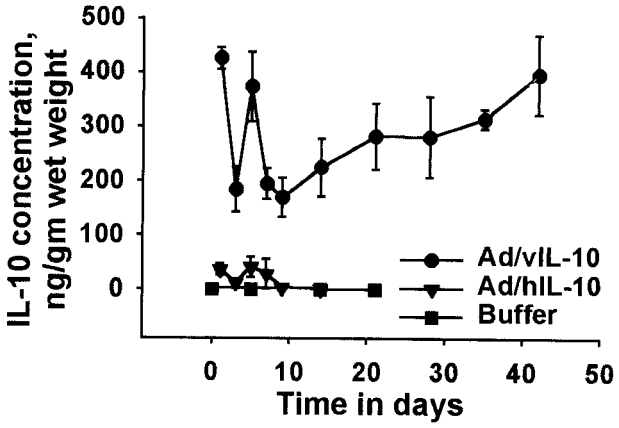

Fig. 1. Lung hIL-10 and vIL-10 expression after i.t. Ad/hIL-10 and Ad/vIL-10 instillation. Following i.t. instillation of 10^{10} particles of either Ad/hIL-10 or Ad/vIL1–0, or buffer alone, vIL-10 expression was increased significantly ($P < 0.001$) and prolonged as compared with hIL-10 levels.

Results

Time Course Response After Ad/hIL-10 and Ad/vIL-10 i.t. Instillation. Mice underwent i.t. instillation of either Ad/hIL-10 or Ad/vIL-10 as described in *Materials and Methods*. Lung vIL-10 levels were increased significantly (10-fold increase) and prolonged as compared with hIL-10 levels ($P < 0.001$), despite a similar dose of administered Ad. In both experimental groups, peak expression was seen on days 1 and 5; however, hIL-10 expression decreased to less than 3% of peak levels by day 14, whereas vIL-10 expression persisted for 42 days without any significant reduction ($P > 0.05$) (Fig. 1). Peak serum levels in mice undergoing i.t. instillation of Ad/hIL-10 and Ad/vIL-10 were not significantly different (0.87 ± 0.10 ng/ml of serum and 2.32 ± 1.04 ng/ml of serum, respectively), with a decrease of these levels to 0.11 ± 0.03 ng/ml of serum 14 days after Ad/hIL-10 delivery, and 0.18 ± 0.06 ng/ml of serum 21 days after Ad/vIL-10 delivery. In addition to the increased and prolonged levels of vIL-10 seen in the lung, the ratio of peak vIL-10 in the lung to vIL-10 in the serum was 13.1 times higher than the ratio of the peak hIL-10 in the lung to hIL-10 in the serum ($P = 0.005$) (Table 1).

IL-1α concentration and myeloperoxidase activity in the lung were increased significantly in animals receiving Ad/hIL-10 as compared with those animals receiving Ad/vIL-10, $P < 0.001$ (Fig. 2 *B* and *C*). TNF-α production in the lung was largely suppressed after delivery of both Ad/hIL-10 and Ad/vIL-10 as

Table 1. Organ IL-10 to serum IL-10 ratios after i.t. and i.v. delivery of Ad/hIL-10 and Ad/vIL-10

	Ratio	
Ad/vIL-10 Treatment group	Lung/serum	Liver/serum
i.t. Ad/hIL-10	36.8 ± 15.0	ND
Ad/vIL-10	481.9 ± 204.4	ND
i.v. Ad/hIL-10	0.03 ± 0.01	0.12 ± 0.05
Ad/vIL-10	0.15 ± 0.01	1.82 ± 0.25

ND, not determined.

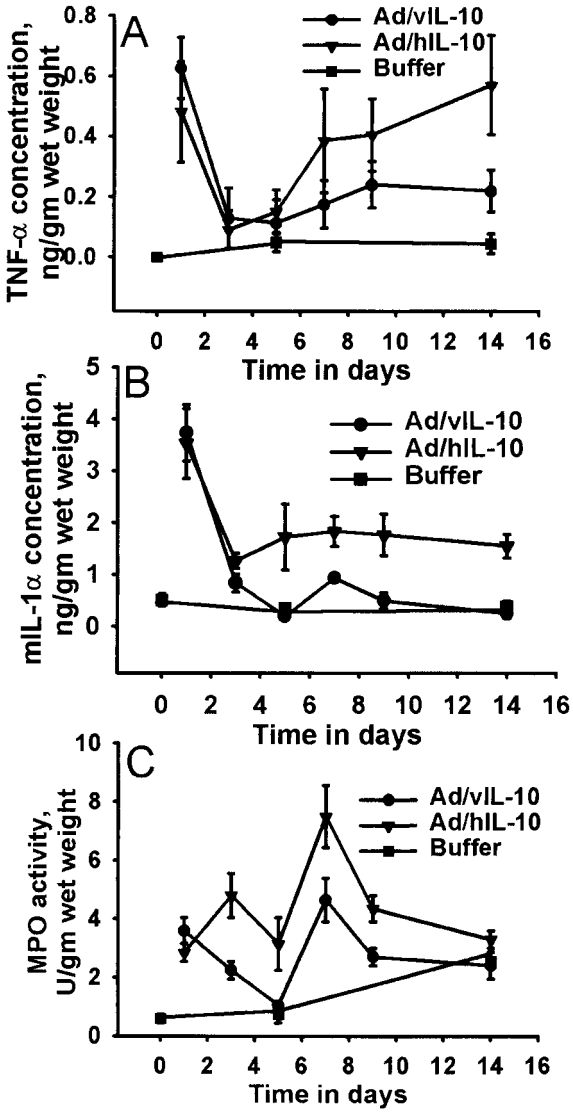

Fig. 2. Lung cytokine appearance after Ad/hIL-10 and Ad/vIL-10 instillation. Lung TNF-α levels were minimal after Ad/IL-10 instillation, but there was a trend toward increased TNF-α production in the lungs of mice receiving Ad/hIL-10 (*A*). Similarly, IL-1α (*B*) and myeloperoxidase (*C*) were increased significantly in the lungs of mice receiving Ad/hIL-10 as compared with those receiving Ad/vIL-10 ($P < 0.001$).

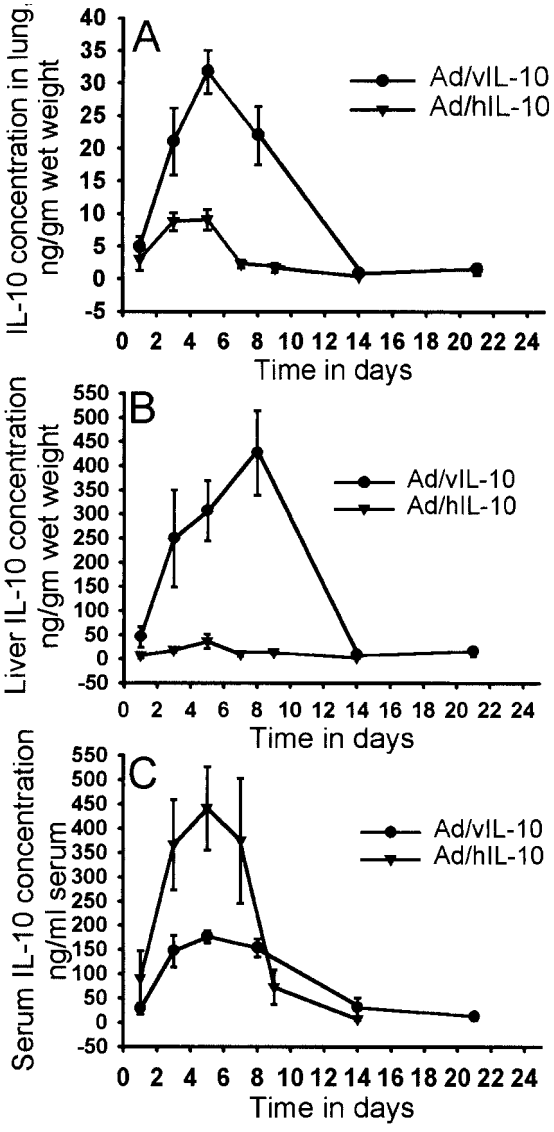

Fig. 3. IL-10 levels after i.v. delivery of Ad/hIL-10 and Ad/vIL-10. After i.v. instillation of 10^{10} particles of Ad/hIL-10 or Ad/vIL1–0, lung and liver vIL-10 levels were increased significantly as compared with hIL-10 levels, $P < 0.001$ (*A* and *B*). Conversely, serum hIL-10 levels were increased significantly over serum vIL-10 levels after i.v. delivery of the Ad/IL-10 constructs, $P < 0.001$ (*C*).

compared with Ad/β-Gal (1.5 $\pm$ 0.4 and 1.4 $\pm$ 0.3 ng/g of wet weight 1 and 7 days after i.t. instillation of Ad/β-gal); however, there was a trend toward increased TNF-α production in the animals receiving Ad/hIL-10 as compared with those receiving Ad/vIL-10 (Fig. 2*A*). Mouse IL-10 was immeasurable in the lungs of these animals.

IL-10 Expression After i.v. Delivery of Ad/hIL-10 and Ad/vIL-10. To further analyze the potential differences in hIL-10 and vIL-10 pharmacokinetics, hIL-10 and vIL-10 expression were evaluated after i.v. delivery of Ad/hIL-10 and Ad/vIL-10. Peak vIL-10 levels again were increased significantly ($P < 0.001$), although not prolonged, as compared with hIL-10 levels in both the livers and lungs of treated mice (10-fold increase in liver and 3-fold increase in lung) (Fig. 3 *A* and *B*), after i.v. delivery. hIL-10 levels, however, were increased significantly ($P < 0.001$) over vIL-10 levels in the serum of treated animals (3-fold increase) (Fig. 3*C*). As seen with the i.t. treated animals, the lung or liver to serum ratios of vIL-10 were increased significantly ($P < 0.001$ for both liver and lung) as compared with the lung or liver to serum ratios of hIL-10 (5.4 times higher for lung and 14.6 times higher for liver) (Table 1).

Pharmacokinetics of Recombinant hIL-10 and vIL-10 Protein. To determine whether a differential rate of clearance of hIL-10 versus vIL-10 could explain the prolonged appearance and/or tissue distribution, mice underwent i.v. instillation of 20 ng/g of body weight of either recombinant hIL-10 or vIL-10. Serum, lung, and liver IL-10 levels were measured at 2, 10, 30, and 60 min. The peak levels of IL-10 achieved at 2 min were 87.2 $\pm$ 9.2 and 67.5 $\pm$ 8.9 ng/ml of serum for hIL-10 and vIL-10, respectively (not significant, $P > 0.05$). There was not a significant difference in the rate of clearance of hIL-10 versus vIL-10 from the serum of these animals (Fig. 4*A*). Interestingly, however, only vIL-10 was measurable in both the lung and liver homogenates of these animals, ranging from 2.7 $\pm$ 0.7 ng/g of wet weight at

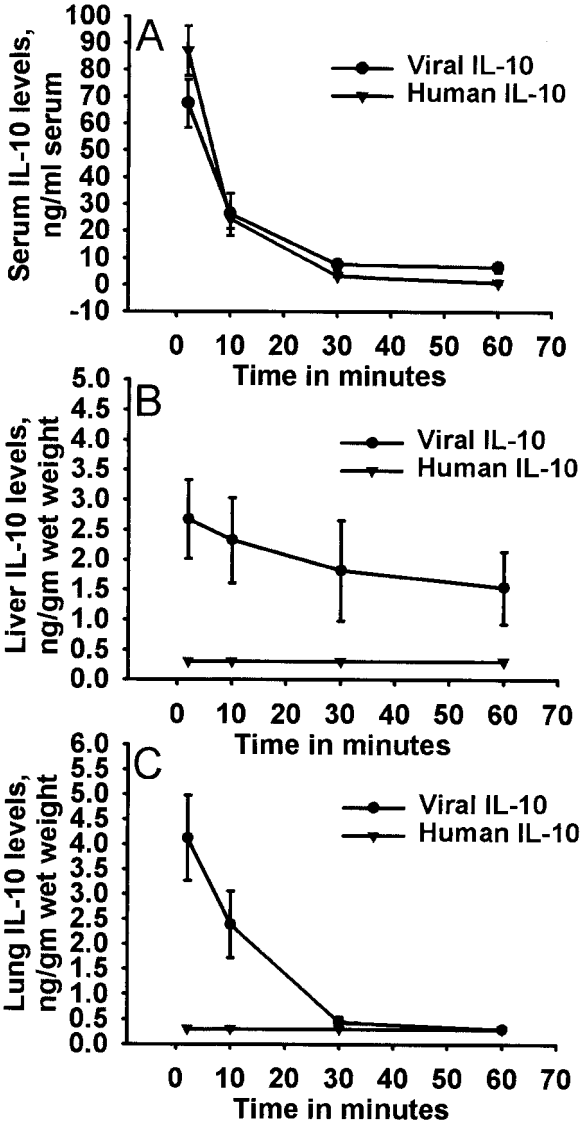

Fig. 4. Intravenous delivery of recombinant hIL-10 and vIL-10. The clearance of recombinant hIL-10 and vIL-10 from the serum of mice after i.v. delivery did not differ significantly (*A*). Only vIL-10 was measurable in the lung and liver after i.v. delivery of the recombinant proteins (*B* and *C*).

2 min to 1.5 ± 0.6 ng/g of wet weight at 60 min in the liver and 4.1 ± 0.8 ng/g of wet weight at 2 min to 0.4 ± 0.1 n g/g of wet weight at 30 min in the lung. In contrast, hIL-10 was immeasurable at all time points in the lung and liver (Fig. 4 *B* and *C*). These data suggest that vIL-10 accumulates at significantly increased levels in tissues as compared with hIL-10.

***In Vitro* Studies.** As lung and liver concentrations of IL-10 were determined from an organ homogenate, and therefore, represent both intracellular and interstitial IL-10 levels, additional *in vitro* studies were undertaken to determine whether there was a significant difference between the fraction of hIL-10 as compared with vIL-10 which was freely secreted into the supernatant as compared with that which was retained in the cell lysate fraction. Cell supernatants and lysates were collected as described after transduction with 10^8, 10^9, and 10^{10} particles of Ad/hIL-10 and Ad/vIL-10. The cell lysate fraction represents intracellular levels of IL-10 as well as any IL-10 that was bound to the cell surface and was released in the lysis process.

Table 2. IL-10 levels after transduction of KD2SV with Ad/hIL-10 or Ad/vIL-10

Treatment group	IL-10		
	Supernatant, ng/ml supernatant	Cell lysate, ng/μg protein	Supernatant/ lysate
1×10^8 Ad/hIL-10	6.72 ± 0.15	0.001	5,509.02
1×10^8 Ad/vIL-10	9.96 ± 0.01	0.027	373.00
1×10^9 Ad/hIL-10	149.25 ± 16.75	0.046	3,223.54
1×10^9 Ad/vIL-10	286.00 ± 3.50	1.823	156.89
1×10^{10} Ad/hIL-10	5,099.50 ± 66.50	4.49	1,137.01
1×10^{10} Ad/vIL-10	9,340.50 ± 1,009.50	66.65	140.13

hIL-10 concentrations in the supernatant fractions were not significantly different from vIL-10 supernatant levels. Although the cell lysate IL-10 levels were minimal as compared with supernatant levels, the vIL-10 cell lysate levels were 25.3 ± 7.1 times higher than the hIL-10 cell lysate levels, as shown in Table 2. To determine whether the proportion of hIL-10 that was freely secreted was increased as compared with vIL-10, the ratios of supernatant/lysate (S/L) IL-10 levels were compared. The S/L ratio for hIL-10 was 15 times greater than the S/L ratio for vIL-10 ($P = 0.002$) (Table 2). This finding suggests that hIL-10 may be more readily secreted from the cell or may have a lower affinity for cell surface binding that occurs exclusive of the IL-10 receptor.

Anti-Ad Antibody Responses After Administration of Ad/hIL-10 or Ad/vIL-10. An alternative explanation for the prolonged appearance of vIL-10 after i.t. instillation might be reduced antibody-mediated clearance of virally infected cells in mice treated with Ad vectors expressing vIL-10 or a differential antibody response against the vIL-10 and hIL-10, as both proteins are foreign in the mouse. Neutralizing anti-Ad antibodies were detected in the serum of mice after i.t. and i.v. instillation of Ad/hIL-10, Ad/vIL-10, and Ad/β-gal. The neutralizing antibody response was decreased significantly in mice receiving both i.t. Ad/hIL-10 and Ad/vIL-10 as compared with Ad/β-gal, 40.1 ± 18.0 and 35.8 ± 8.5 versus 126.1 ± 26.4 (inverse titer) respectively, $P <$ 0.001, and was not increased significantly as compared with buffer-treated animals (Fig. 5*A*). Similarly, neutralizing anti-Ad antibody titers were decreased significantly in the mice receiving i.v. Ad/hIL-10 or Ad/vIL-10 as compared with Ad/β-gal, 71.3 ± 48.2 and 64.2 ± 27.4 versus 313.5 ± 114.1, respectively ($P <$ 0.001), and again were not elevated significantly as compared with buffer-treated animals (Fig. 5*B*). Similarly, there was only a minimal antibody response against both hIL-10 and vIL-10 proteins after i.t. administration of Ad/hIL-10 and Ad/vIL-10, whereas there was a significant antibody response against β-gal after i.t. delivery of Ad/β-gal (Table 3). Thus, it seems unlikely that antibody-mediated cytolysis of Ad-infected cells or antibody clearance of the IL-10 vectors can explain the differential clearance of vIL-10 and hIL-10 after i.t. Ad instillations.

Discussion

Ad IL-10 gene therapy has been widely explored for the treatment of many disease processes *in vivo*, including cardiac and hepatic transplantation, rheumatoid arthritis, bronchiolitis obliterans, and endotoxemia (16–21). However, much of our knowledge concerning the biology of hIL-10 and vIL-10 thus far has been derived from *in vitro* systems. In the present report, we have explored tissue levels, distribution, and biological responses of vIL-10 and hIL-10 expressed with first-generation Ad vectors. These vectors contain deletions in both the E1 and E3 regions. Deletions in the E3 region, specifically E3 14.7- and 19-kDa genes, have been associated with an exacerbation in pulmonary

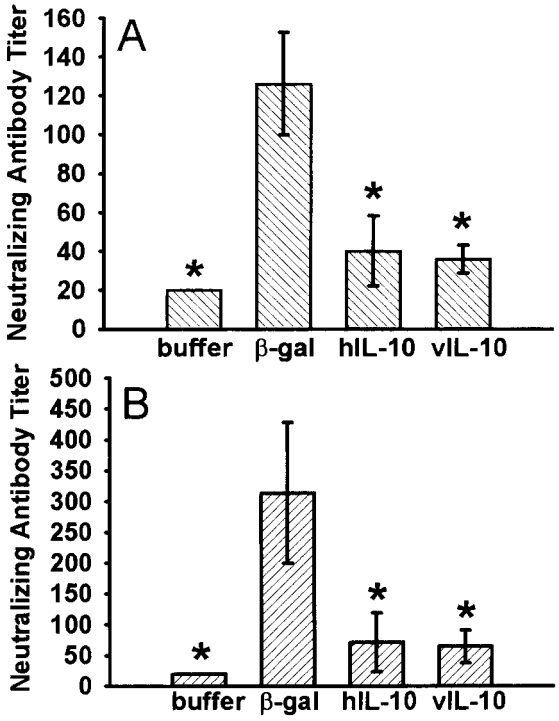

Fig. 5. Serum neutralizing anti-Ad antibody levels after i.t. and i.v. delivery of Ad/β-gal, Ad/hIL-10, or Ad/vIL-10. Anti-Ad antibody levels were reduced significantly in mice receiving either i.t. or i.v. Ad/hIL-10 and Ad/vIL-10, as compared with mice receiving Ad/β-gal, $P < 0.001$. The anti-Ad antibody levels that developed in both the i.t. (*A*) and i.v. (*B*) Ad/hIL-10- and Ad/vIL-10-treated animals were not increased significantly over the levels seen in mice treated with buffer alone. Buffer-treated animals had immeasurable antibody levels and were conservatively given a titer of 20, the lowest dilution factor measured.

inflammation secondary to an increased cytotoxic cellular response and increased pulmonary neutrophil infiltration. (22, 23). At the doses used in these studies (10^{10} particles), however, the E1/E3-deleted Ad vectors expressing IL-10 caused only minimal neutrophil infiltration as demonstrated by the myeloperoxidase content in Fig. 2*C*. Although the data are not presented in this study, we have treated cotton rats (*Sigmodon hispidus*) with similar doses of these adenoviral constructs (10^{10} particles) and have seen only minimal histological evidence of inflammation. Animals receiving Ad vectors expressing vIL-10 actually had reduced evidence of inflammation compared with animals receiving vectors expressing hIL-10, which had a very modest bronchiolitis (R.M.M. and L.L.M., unpublished data).

Despite an 84% amino acid sequence identity between hIL-10 and vIL-10 (6, 7) and shared signaling through the same IL-10 receptor (8), significant differences were observed for hIL-10 and vIL-10 accumulation and tissue distribution after Ad delivery. vIL-10 levels were significantly higher in tissue homogenates, regardless of the route of delivery (i.t. or i.v.) as compared with hIL-10. In contrast, serum concentrations were either similar or greater in mice transfected with Ad vectors expressing hIL-10 rather than vIL-10. Moreover, when recombinant hIL-10 and vIL-10 protein were administered intravenously, only vIL-10 was measurable in the lung and liver homogenates of injected mice despite a similar clearance from the blood. These latter findings suggest that the clearances of the two proteins are mediated by two distinct processes; hIL-10 and vIL-10 are cleared from the blood with similar pharmacokinetics, whereas vIL-10 appears to be retained in tissue compartments.

Table 3. Total transgene-specific immunoglobin levels after i.t. delivery of Ad/β-gal, Ad/hIL-10, or Ad/vIL-10

Virus delivered i.t.	Level, ng/ml	
	IgG	IgM
Ad/β-gal	$1.6 \times 10^8 \pm 0.15 \times 10^8$	19,103 ± 7,735
Ad/hIL-10	18 ± 5.6	35 ± 11
Ad/vIL-10	68 ± 45	100 ± 52

These results seem to be consistent with the *in vivo* Ad transduction studies. Intravenously administered Ad vectors result in preferential expression in the liver (24). In the present studies, IL-10 concentrations in the liver and lung were markedly higher after i.v. delivery of Ad/vIL-10 than after Ad/hIL-10, whereas serum hIL-10 levels were increased markedly compared with vIL-10. We presume that the majority of IL-10 measured in the serum was derived from tissues such as the liver and released into the systemic circulation. The *in vitro* transduction studies are consistent with this conclusion. When KD2SV cells were transduced with Ad vectors expressing hIL-10, the ratio of secreted to cell lysate protein was significantly higher than was seen in cells transduced with Ad vectors expressing vIL-10. Therefore, the significantly higher serum levels of hIL-10 in animals receiving i.v. Ad/hIL-10 are attributable, at least in part, to increased release of hIL-10 into the serum as compared with vIL-10. However, there are clearly other mechanisms involved as well, because the recombinant vIL-10 was detectable in organs after i.v. administration and hIL-10 was not. Together these results suggest that sequence-based structural differences in vIL-10 may affect accumulation and clearance in various organs.

The prolongation of vIL-10 appearance in the lung after i.t. delivery of Ad/vIL-10 as compared with hIL-10 was unexpected. In fact, most studies have reported duration of protein expression after i.t. delivery of Ad vectors to be less than 21 days. In our hands, expression of both β-gal and hIL-10 had declined to less than 5% of peak levels within 14 days, whereas there was no significant decline in vIL-10 levels 42 days after i.t. instillation of Ad vectors. These data were confirmed by using real-time reverse transcription-PCR analysis of mRNA copy number. Between days 5 and 14, the number of hIL-10 transcripts in the lungs declined by 79%, whereas the number of vIL-10 transcripts remained unchanged (+3%) (data not shown).

One explanation for the differential tissue distribution between Ad vectors expressing hIL-10 and vIL-10 may be the 1000-fold higher affinity for the mouse IL-10 receptor that hIL-10 possesses as compared with vIL-10 (8). It is possible that hIL-10 is being rapidly internalized and degraded by receptor-mediated endocytosis because of this increased binding affinity for the IL-10 receptor. Another possibility is that vIL-10 has the ability to bind to the extracellular matrix (ECM) or to cell surface proteoglycans, whereas hIL-10 cannot. The ECM has been shown to play a central role in a number of cytokine and growth factor activities (25, 26), and Lortat-Jacob *et al.* (27) have demonstrated that IFNγ displays a high affinity for heparan sulfate on the ECM and the surface of some cells, in addition to its cellular receptor. This is one possible mechanism that could explain the increased levels of vIL-10 that we found associated with the vIL-10 transduced cell lysate fractions, as well as the vIL-10 accumulation in the lung and liver seen after administration of the recombinant protein.

In contrast, it is unlikely that the difference in lung IL-10 appearance between i.t. instillation of Ad/vIL-10 and Ad/hIL-10 could be explained by differences in the anti-Ad acquired immune response, because antibody responses to both Ad proteins and the transgenes (hIL-10 and vIL-10) were similarly suppressed in animals receiving i.t. Ad/hIL-10 and Ad/vIL-10.

Furthermore, animals administered Ad/β-gal had a significant antibody response against the viral proteins and β-gal, yet duration of expression was not markedly different from mice receiving Ad/hIL-10.

Rather, one potential explanation for the prolonged appearance of vIL-10 after i.t. instillation of the Ad vectors is the inhibition of the innate immune response versus Ad vectors expressing β-gal or hIL-10. We and others reported that clearance of Ad vectors from the lung was secondary to activation of the innate immune system, primarily by the proinflammatory cytokine TNF-α (28). Expression of Ad vectors is markedly enhanced (9), and in some cases duration of expression is prolonged (29, 30) in mice lacking a functional TNF-α or TNF receptors. Although both vIL-10 and hIL-10 expression reduced TNF-α production in the lungs, vIL-10 expression also reduced neutrophil infiltration and IL-1α appearance, whereas hIL-10 expression appeared to increase these measures of innate immune activation, as compared with vIL-10. These latter findings are consistent with the differing inflammatory profiles that hIL-10 and vIL-10 possess (2) and suggest that activation of the innate immune system is one of the determinants of Ad gene expression.

The *in vivo* properties of vIL-10 as demonstrated here have significant clinical potential. First, the prolonged expression of vIL-10 after i.t. instillation and the absence of a significant acquired immune response suggest that Ad vectors expressing vIL-10 may be applicable to chronic inflammatory diseases in the lung, such as cystic fibrosis or chronic pulmonary fibrosis. The potential to suppress innate immune responses and prolong its own expression in the lung makes vIL-10 an attractive therapeutic tool not only for acute but also for chronic lung inflammation.

Second, the propensity for Ad vectors expressing hIL-10 to result preferentially in elevated blood concentrations when delivered systemically makes it unattractive as an agent for targeted expression in individual tissues. Localized tissue expression after systemic delivery cannot be achieved easily without the potential complications of systemic responses to IL-10. In contrast, Ad vectors expressing vIL-10 appear to result in high tissue levels of protein expression predominantly, suggesting a more efficient means to target expression without the detriments of high systemic levels of IL-10. Further studies will be necessary to determine whether these differential expression patterns can be used for the treatment of acute and chronic inflammatory diseases.

We thank Dr. Kevin Moore (DNAX, Inc.) for his insightful advice and the viral and human cDNA constructs. This work was supported in part by Grant P30 HL-59412, awarded by the National Heart, Lung and Blood Institute, GM-40586, awarded by the National Institute of General Medical Sciences, and a contract with Canji, Inc. R.M.M. is currently supported by a research fellowship (T32-GM-08721) in burns and trauma, awarded by the National Institute of General Medical Sciences.

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